Validation of p16^{INK4a} as a Marker of Oncogenic Human Papillomavirus Infection in Cervical Biopsies from a Population-Based Cohort in Costa Rica

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Abstract

Due to the high prevalence of cancer-associated types of human papillomavirus (HPV) and the poorly reproducible histologic classification of low-grade lesions, identifying infected women at highest risk for cancer prior to neoplastic progression remains a challenge. We therefore explored the utility of p16^{INK4a} immunostaining as a potential diagnostic and prognostic biomarker for cervical neoplasia using paraffin-embedded tissue blocks (punch biopsies and loop electrosurgical excision procedures) obtained from women referred to colposcopy during the enrollment phase of the Guanacaste Project (1993 to 1994). All blocks from 292 women selected by HPV status (HPV negative, nononcogenic HPV positive, or oncogenic HPV positive) and representing the diagnostic spectrum of the population [normal to precancer: cervical intraepithelial neoplasia (CIN) 3] were immunostained for p16^{INK4a} using the p16^{INK4a} research kit based on the monoclonal antibody clone E6H4 (MTM Laboratories, Heidelberg, Germany). For CIN3, the sensitivity of diffuse p16^{INK4a} immunostaining was 100% and the specificity was 95%. For CIN2, the sensitivity and specificity for diffuse staining were 81.1% and 95.4%, respectively. Generalized to the 10,000-woman cohort, this translated to positive predictive value and negative predictive value of 13.9% and 100% for CIN3, respectively, and 20.4% and 99.7% for CIN2 or CIN3, respectively. Of women with an initial diagnosis of less than CIN2 for whom follow-up data for up to 5 to 7 years were available, 44% with diffuse staining developed persistent infection (CIN2 or CIN3). Whereas our data support the diagnostic potential for p16^{INK4a}, further prospective studies with detailed follow-up determining the prognostic capacity of this marker are needed. (Cancer Epidemiol Biomarkers Prev 2004;13(8):1355–60)

Introduction

Infection with 1 of ~15 oncogenic types of human papillomavirus (HPV) is a necessary but insufficient cause of cervical neoplasia (1). However, most HPV infections including those involving oncogenic types remit spontaneously, especially among young women

(2). Therefore, HPV is a sensitive marker for identifying patients at risk for cervical neoplasia, but it has relatively weak positive predictive value (PPV) for identifying women (particularly those ages <30 years) with prevalent cancer precursor. The histologic classification of HPV-induced low-grade lesions called cervical intraepithelial neoplasia (CIN) 1 is also very heterogeneous and poorly reproducible (3). Accordingly, the development of a biomarker that could distinguish which HPV-infected women were at greatest risk for progression to cervical neoplasia would be very useful.

A fundamental characteristic of cervical cancer precursors is that the cells express two oncogenic HPV proteins, E6 and E7, which promote the degradation of human p53 and Rb proteins (4). This process activates a negative transcriptional feedback loop that results in strong overexpression of cyclin-dependent kinase inhibitor p16^{INK4a}. Because early dysplastic lesions with or without deregulated viral E6-E7 oncogene expression might both manifest histologically as low-grade (CIN1) lesions, specific markers that differentiate low-grade dysplastic lesions with or without deregulated viral

oncogene expression might help identify low-grade cervical lesions with increased risk for HPV persistence and neoplastic progression.

High levels of $p16^{INK4a}$ have been showed in both HPV-transformed cell lines and human cervical tumors (4, 5). Theoretically, $p16^{INK4a}$ represents a promising biomarker, because its expression reflects both that oncogenic HPV is present and that it has disrupted normal cell cycle function. Studies to date suggest that diffuse $p16^{INK4a}$ immunostaining of cervical tissue using an anti-$p16^{INK4a}$ monoclonal antibody might be a useful diagnostic marker of oncogenic HPV infections in a subset of definite CIN1 or more severe lesions (5-7). More recently, similar demonstrations of $p16^{INK4a}$ staining of dysplastic cells in cytologic specimens or cervical smears have been reported (8-10). Despite the growing evidence that $p16^{INK4a}$ may be a promising biomarker for cervical lesions with deregulated viral oncogene expression, its sensitivity, specificity, PPV, and negative predictive value (NPV) and its use as a prognostic marker of cervical progression have not been established in a large epidemiologic study (11). We therefore sought to validate $p16^{INK4a}$ as a biomarker of oncogenic HPV infection and cervical neoplasia in the Guanacaste Project, a population-based natural history study of cervical neoplasia in Costa Rica. We assessed the feasibility of $p16^{INK4a}$ immunostaining, calculated the population-based screening characteristics of $p16^{INK4a}$, and assessed the prognostic values of the biomarker based on available follow-up data within the cohort.

Methods

Study Population. This study was nested within an ongoing population-based cohort study of 10,049 women in Guanacaste, Costa Rica (12, 13). Study enrollment was conducted in 1993 to 1994 with the approval of the National Cancer Institute (NCI) and local institutional review boards; all participants provided written informed consent. Briefly, the cohort encompasses a representative sample of the adult female population of Guanacaste, Costa Rica, based on selection by cluster sampling. Women were screened using three cytologic and one visual test at enrollment; colposcopy referral with biopsy of visible lesions was done for any abnormal or equivocal screening results. Cold-knife conization or loop electrosurgical excision procedures (LEEP) were done for treatment of high-grade disease.

Inclusion Criteria. Women (n = 542) from whom paraffin-embedded histologic blocks were collected at enrollment were considered eligible for the present analysis. Subjects included in the current analysis were selected from among eligible women stratified by cytologic and histologic interpretation and HPV status. In selecting our study population, women were stratified into three groups based on their cervical HPV result (oncogenic HPV positive, nononcogenic HPV positive, or HPV negative). Within the three HPV strata, we aimed to select a representative group of women within each of the diagnostic spectra (normal, equivocal, CIN1, CIN2, and CIN3). Equivocal diagnosis included those diagnosed by cervigram, conventional Papanicolaou smear or ThinPrep Pap; CIN1 diagnosis included low-grade intraepithelial lesions. Because CIN1 was our main group of interest, we oversampled from the available strata, selecting virtually all women (90%) diagnosed with CIN1 regardless of HPV status. In addition, we designed our analysis to test the specific delineation between oncogenic and nononcogenic HPV infection and therefore included virtually all nononcogenic HPV positive women with low-grade disease outcomes. Last, we also oversampled rare strata such as HPV-negative high-grade lesions. In all, we aimed to select ~100 women from each HPV strata and resulted in a total of 311 women for the present analysis.

All blocks for each woman were retrieved for $p16^{INK4a}$ immunostaining. Because LEEPs yielded multiple blocks, the final number of blocks and resulting slides exceeded the number of cases.

Exclusion Criteria. Of the 311 women initially selected, 13 women (with a total of 96 slides) were excluded because the amount of squamous epithelium was inadequate for $p16^{INK4a}$ staining. It is important to note that slides were made from all blocks in a given procedure; for these 13 women, their slides originated from LEEP procedures (~4 to 6 blocks per woman). The small nature of these particular lesions resulted in insufficient tissue for analysis. Another six women (13 slides) were further excluded due to inadequate $p16^{INK4a}$ staining.

Final Population. Our final analytic population of 292 women (619 slides) included 58 women with a normal diagnosis, 121 equivocal, 75 CIN1, 19 CIN2, and 19 CIN3. Of the 292 women, 231 had one slide and 61 had multiple slides (range 2-17; Table 1).

Table 1. Final representative Costa Rican population selected from 542 women with biopsy or LEEP by diagnosis at enrollment stratified by HPV status (Oncogenic HPV positive, nononcogenic HPV positive, or HPV negative)

Diagnosis	Oncogenic HPV positive (n = 190), Selected/Total (%)	Nononcogenic HPV positive (n = 74), Selected/Total (%)	HPV negative (n = 278), Selected/Total (%)	Total (n = 542), Selected/Total (%)
Normal	18/19 (95)	13/13 (100)	27/83 (33)	58/115 (50)
Equivocal	31/33 (94)	39/41 (95)	51/161 (32)	121/235 (51)
CIN1	44/48 (92)	13/13 (100)	18/22 (82)	75/83 (90)
CIN2	9/31 (29)	4/4 (100)	6/7 (86)	19/42 (45)
CIN3	11/59 (19)	3/3 (100)	5/5 (100)	19/67 (28)
Total	113/190 (59)	72/74 (97)	107/278 (38)	292/542 (54)

NOTE: Total indicates the total number of possible cases biopsied, whereas Selected indicates the number of women within the strata that were selected for $p16^{INK4a}$ immunostaining.

p16^INK4a Staining Methods. Immunostaining of cervical biopsies was done according to the manufacturer's instructions using the p16^INK4a research kit (MTM Laboratories, Heidelberg, Germany), which is based on the primary monoclonal antibody clone MTM-E6H4. The p16^INK4a research kit is a qualitative immunohistochemical assay for the evaluation of overexpressed cyclin-dependent kinase inhibitor p16^INK4a antigen on formalin-fixed, paraffin-embedded slides from cervical biopsies. Two sections (5 μm thickness) of each block were cut and used for this study, one for a H&E staining slide and the other for p16^INK4a staining. The MTM pathologist (M.T.) diagnosed the conventionally stained slide and the p16^INK4a stained slide. The MTM pathologist remained blinded to the final Costa Rican diagnosis from which the block selections were made and from which the p16^INK4a stained slides were ultimately compared. H&E readings from the MTM pathologist were also used to assess agreement with the original diagnosis made for the cohort (M.E.S.).

p16^INK4a immunostains were scored as negative, sporadic, focal, and diffuse by the staining pattern; they were further categorized by strength of staining (0 to 3+) and distribution of staining (none to whole epithelium). For each woman, a single p16^INK4a reading was used for our subsequent analyses. For 61 women with multiple p16^INK4a readings due to the multiple blocks submitted (range 2 to 17 slides), the most severe p16^INK4a reading was used. H&E stained slides were categorized as cervicitis, immature metaplasia, mature metaplasia, atypical metaplasia, CIN1, CIN2, CIN3, and invasive carcinoma. A NCI pathologist (M.E.S.) read 10% of the MTM H&E slides to assess agreement (>90%) and remained blinded to both MTM diagnosis and final diagnosis set by Costa Rica. Examples of p16^INK4a immunostained slide paired with the H&E slides in the Costa Rica specimens are shown in Fig. 1.

HPV DNA Testing. Cervical cytologic specimens were tested for HPV DNA using Gold Taq and the L1 MY09/MY11 consensus primer methods (14, 15). For this analysis, oncogenic or high-risk HPV was considered positive for HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 68. Nononcogenic or low-risk HPV was defined as positive for HPV 6, 11, 13, 26, 32, 34, 40, 42, 43, 44, 53, 54, 55, 57, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73, 74, 81, 83, 84, or 85. HPV negative was defined as PCR negative for all detectable types.

Statistical Methods. Although p16^INK4a staining was initially coded in four distinct categories, subsequent published analysis have strongly suggested the lack of significant distinction between sporadic and focal staining (16). Therefore, for all subsequent analyses, we have combined sporadic and focal staining into a single group. The number and percentage of women with each of the three p16^INK4a staining result categories (negative, sporadic/focal, and diffuse) were calculated in each diagnostic category (normal, equivocal, CIN1, CIN2, and
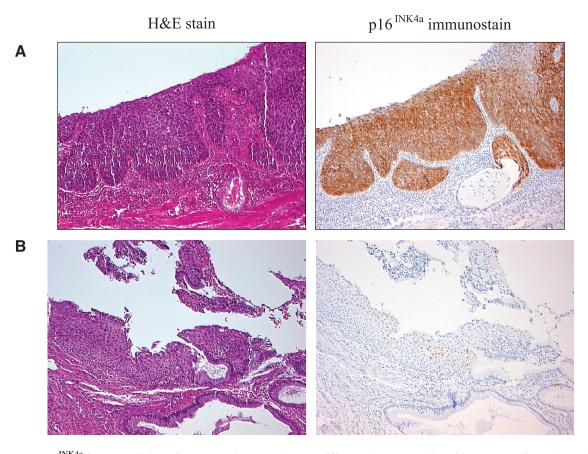

Figure 1. p16^INK4a immunostaining of representative samples, (**A**) diffuse and (**B**) sporadic, with corresponding H&E stain.

Table 2. p16^INK4a immunostaining in cervical tissues from representative sample of population from Costa Rica (Diagnosis based on both cytology and histology)

Diagnosis (n = 292)	HPV status	Negative (n = 139)	Sporadic/ Focal (n = 83)	Diffuse (n = 70)
Normal (n = 58)	HPV negative (n = 27)	19 (70)	7 (26)	1 (4)
	Nononcogenic positive (n = 13)	10 (77)	3 (23)	0 (0)
	Oncogenic positive (n = 18)	10 (56)	6 (33)	2 (11)
Equivocal (n = 121)	HPV negative (n = 51)	36 (71)	14 (27)	1 (2)
	Nononcogenic positive (n = 39)	25 (64)	13 (34)	1 (3)
	Oncogenic positive (n = 31)	16 (52)	8 (26)	7 (23)
CIN1 (n = 75)	HPV negative (n = 18)	10 (56)	7 (39)	1 (6)
	Nononcogenic positive (n = 13)	3 (23)	7 (54)	3 (23)
	Oncogenic positive (n = 44)	8 (18)	13 (29)	23 (52)
CIN2 (n = 19)	HPV negative (n = 6)	1 (17)	0 (0)	5 (83)
	Nononcogenic positive (n = 4)	1 (25)	1 (25)	2 (50)
	Oncogenic positive (n = 9)	0 (0)	4 (44)	5 (56)
CIN3 (n = 19)	HPV negative (n = 5)	0 (0)	0 (0)	5 (100)
	Nononcogenic positive (n = 3)	0 (0)	0 (0)	3 (100)
	Oncogenic positive (n = 11)	0 (0)	0 (0)	11 (100)

CIN3). The sensitivity and specificity of p16^INK4a as a biomarker of HPV and histologic status were calculated for the entire Costa Rican cohort. This was done by using data from the current analysis to reconstitute the entire study population based on our sampling fraction for each of our selected groups (by HPV status and histology). The two possible cut points of diffuse versus nondiffuse (focal/sporadic and negative) and diffuse and focal/sporadic versus negative p16^INK4a staining were assessed. The sensitivity of identifying oncogenic HPV positive CIN3 and the specificity of HPV negative normal diagnosis were of particular interest. Analysis restricted to disease classified by histology only was also done.

Using available follow-up data from the cohort (up to 7 years), the predictive values of p16^INK4a immunostaining for CIN3, CIN2, HPV persistence, and HPV clearance were calculated for those women diagnosed at enrollment with CIN1 or less. HPV persistence is defined as HPV positive for the same oncogenic HPV type at enrollment and at the time of diagnosis, which on average was at 5 years of follow-up.

Agreement between MTM and NCI diagnosis was assessed. The MTM result was compared with the data existent from the histopathology diagnoses already assigned to each case by the study pathologists in Costa Rica and NCI. For women with available follow-up data, all H&E sections from the original diagnostic tissue blocks were also re-reviewed by the study pathologist

(M.E.S.) to ensure that no misclassification of the original block diagnosis occurred. In addition, agreement of p16 immunostaining between multiple slides (from the 61 women with multiple blocks) was also assessed.

Results

Of the 10,049 women enrolled in the Costa Rican cohort from 1993 to 1994, 542 women had biopsy or a LEEP at enrollment. Our final sample of 292 women consisted of 113 oncogenic HPV positive DNA, 72 nononcogenic HPV positive DNA, and 107 HPV negative DNA (Table 1). Unless otherwise specified, all references to tissues represent a staining result for a single woman. By diagnosis, 3 (5%) women with normal tissue, 9 (7%) women with an equivocal diagnosis, and 27 (36%) women with CIN1 diagnoses stained diffusely. On the contrary, 12 (63%) CIN2 and 19 (100%) CIN3 diagnoses stained diffusely.

The effect of increasing p16^INK4a immunostaining with increasing disease severity is showed in Table 2. A clear gradient is observed across the diagnostic categories with a predominance of negative and sporadic/focal staining in women with normal and equivocal diagnosis and a predominance of diffuse staining in those with a CIN2 or CIN3 diagnosis. Specifically, of women with a normal diagnosis, only 1 (4%) HPV negative, 0 (0%) nononcogenic HPV positive, and 2 (11%) oncogenic HPV positive tissues stained diffusely. This is in contrast to women diagnosed with CIN3 in which, regardless of HPV status, 100% of tissues stained diffusely for p16^INK4a. Although an increasing percentage of oncogenic HPV positive women with equivocal, CIN1, or CIN2 diagnosis stained diffusely for p16^INK4a, staining was predominantly associated with increasing severity of the histologic diagnosis.

Based on the two distinct cut points for p16^INK4a immunostaining, we calculated the screening characteristics (sensitivity, specificity, PPV, and NPV), expanding our calculations to the Costa Rican cohort based on the selected sampling fraction. The cut point of sporadic or greater p16^INK4a staining revealed a sensitivity of 97% for a CIN2 or greater outcome and a specificity of 68.8% (Table 3). The sensitivity declined for the more stringent cut point of diffuse staining (81.1%); however, the specificity increased to 95.4%. The NPVs for all cut points exceed 99%, and the PPVs increased from 4.4% for the sporadic or greater staining to 20.4% for diffuse immunostaining. For a CIN3 outcome, the sensitivity of p16^INK4a immunostaining was 100% for both cut points; the specificity increases from 68.4% with a sporadic or greater cut point to 95.0% for diffuse p16^INK4a immunostaining.

Table 3. Sensitivity, specificity, PPV, and NPV of p16^INK4a immunostaining in the Costa Rican population

p16^INK4a Cut point	Outcome	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Sporadic+	CIN2+	97.0	68.8	4.4	99.9
Diffuse	CIN2+	81.1	95.4	20.4	99.7
Sporadic+	CIN3	100.0	68.4	2.5	100.0
Diffuse	CIN3	100.0	95.0	13.9	100.0

The PPV increased from 2.5% for a sporadic or greater cut point to 13.9% for diffuse immunostaining.

For those women initially diagnosed as normal to CIN1 (n = 254), 199 had follow-up data for up to 5 to 7 years. For these women, 8 of 18 with diffuse p16[INK4a] immunostaining developed persistent oncogenic HPV infection (n = 1), CIN2 (n = 4), or CIN3 (n = 3) for a PPV of 44%. For CIN2 or CIN3, a PPV of 39% (7 of 18) was observed. Of the 181 without diffuse staining at enrollment, 153 cleared HPV infection for a NPV of 85%. Further analyses by length of follow-up did not alter the PPV or NPV; although immunostaining identified women who developed CIN3 after 5 years, a negative stain was observed for some women who developed CIN3 within 1 year. Nevertheless, the risk for CIN progression or HPV persistence remains higher for women with diffuse staining for p16[INK4a] compared with those without diffuse staining at enrollment.

Discussion

To assess the utility of p16[INK4a] as a biomarker for triage, our primary goal, we assessed with particular interest those lesions that were CIN1 or had a less severe diagnosis. We believed that for the biomarker to be considered successful, it should stain diffusely those blocks representing oncogenic HPV infections that have produced CIN1+ lesions destined to progress but not stain diffusely nononcogenic infections or disease less than CIN1 and likely to regress. Although both HPV typing and histologic grading are prone to some error, within those constraints, a biomarker would still possess a very strong association of staining with the definite oncogenic lesions. We further increased the confidence of our specificity estimates by including totally normal blocks.

Our results show good correlation between p16[INK4a] immunostaining and cervical disease severity stratified by HPV status. Consistent with studies to date, all HPV positive high-grade (CIN3) tumors in the present study were positive for p16[INK4a] expression. Sensitivity of diffuse staining for CIN3 and its specificity for HPV negative normal tissues are both high based on our cross-sectional data. Although our study shows that p16[INK4a] immunostaining accurately predicts CIN3, we did not find it identifying all CIN2 as reported previously (16). Nevertheless, several HPV negative CIN2 did stain diffusely for p16[INK4a], and differences with previous studies are likely attributable to differential interpretation between pathologists and/or studies in what is categorized as CIN2.

Consistent with the original publication of Klaes et al. (5) of 272 women based on histology and cytology specimens, all high-grade cervical lesions in our study showed high levels of p16[INK4a] expression, supporting the principle that p16[INK4a] identifies CIN2 or CIN3. In the recent study by Agoff et al. (7) of 569 women, p16[INK4a] expression was also shown to correlate with increasing severity of cervical disease.

Hypothetically, p16[INK4a] expression delineates oncogenic and nononcogenic HPV types (17). The Sano et al. (6) study of 56 histologic specimens showed distinct delineations between p16[INK4a] staining and oncogenic HPV types; Klaes et al. (16) also showed a clear delineation of p16[INK4a] overexpression for nononcogenic and oncogenic HPV types within low-grade lesions such as CIN1, showing expression to be restricted to oncogenic HPV types. However, our results were similar to those by Keating et al. (18) in which p16[INK4a] was correlated to oncogenic HPV but did not show a distinct delineation. For our current study, this may be attributed to the lack of HPV testing in the tissue; our HPV typing was based on cervical samples and thus subject to potential misclassification.

Our secondary goal of the present analysis was to assess the screening characteristics of p16[INK4a]. Keating et al. (19) reported screening characteristics for p16[INK4a] based on 85 histologic specimens and found a high PPV for p16[INK4a] and any lesions (both low-grade intraepithelial lesion and high-grade intraepithelial lesion). In our study, the sensitivity and specificity of p16[INK4a] are high particularly when we reconstituted the Costa Rican cohort based on our sampling fractions. The ability to apply p16[INK4a] immunostaining in exfoliated cytology specimens will be important for the widespread use of p16[INK4a] in cervical neoplasia for screening purposes. A growing number of studies have showed the relationship between p16[INK4a] immunostaining in cytology specimens collected using a variety of methods (8-10). Our limited numbers of multiple slides support this notion of a potential effect by increased intensity of staining over the increased number of cells. We simulated the field effect of immunostaining with a subanalysis of those women who had multiple slides. Although we found that p16[INK4a] staining varied considerably among LEEP slides, there was an association between the number of slides staining positive with severity of diagnosis; thus, cytology-based staining that represents an average of diffuse, focal/sporadic staining of the cervix might prove promising.

Last, to determine its utility as a prognostic marker, we believe that predictive values based on the cross-sectional data and the prospective data are particularly important for the interpretation of these data. The predictive values were modest in our small number of women for whom follow-up data were available, and time to disease progression did not necessarily inform our analysis; we found that diffuse staining predicted some women developing CIN3 after 5 years of enrollment but also missed some women who developed CIN3 in the following year. This could be attributed to misclassification of diagnosis in the original block, which was no longer available for sectioning. However, it is also conceivable that, although p16[INK4a] may not detect rapidly progressing lesions that do not yet display deregulated viral expression at the time of measurement, it does seem to delineate those lesions less likely to regress.

Strengths of our study include the large sample of women with equivocal and low-grade (CIN1) lesions, the main nondisease group of interest. Our selection by HPV status further allowed the assessment of disease and infection range for which to assess p16[INK4a] expression. Study strengths also include small biopsies, including small high-grade lesions, which are characteristic of the study population; this provided a greater opportunity to assess the ability of p16[INK4a] to reflect acute infections and to discriminate between low-grade lesions. We also

made every attempt to obtain objective p16^{INK4a} results by blinding the pathologist performing the p16^{INK4a} staining to our original disease diagnoses; however, there still exists the inherent bias that a pathologist would determine diagnoses based on the sections received for p16^{INK4a} staining.

The final diagnosis for the Costa Rican cohort at enrollment was based on a combination of cytology and histology readings. Because p16^{INK4a} immunostaining is based on histology samples, we also conducted analyses restricted to histologic diagnosis only; however, this restriction did not alter our results. It is of importance, however, that although some CIN2 and CIN3 diagnoses were based on cytology and histology, p16^{INK4a} stained those diagnoses diffusely. Our HPV typing is subject to limitations, as HPV typing was based on cervical swabs and not the tissue demonstrating the lesion. Therefore, our HPV negative CIN3 strata are likely misclassified for HPV status. In addition, HPV testing was completed for specimens from the enrollment examination that preceded biopsy or LEEP by a few months on average. This misclassification likely accounts for the lack of overall association between HPV positivity and p16^{INK4a} staining.

Study limitations also include the resulting sample size, although this is a result of a 10,000-woman cohort. Our extrapolation of sensitivity, specificity, and PPV to the entire population is also subject to some limitations. Although random selection within each strata was conducted, our extrapolation to the population as a whole will likely be biased by the inclusion of typically rare strata in a general population, such as women with normal diagnosis who would typically not have biopsy specimens available and women in the HPV negative CIN3 strata, which is likely due to misclassification from HPV typing as indicated previously. Nevertheless, the high level of specificity for normal diagnosis, despite this bias and use of women referred for colposcopy due to abnormal Papanicolaou smear as our normal comparison group, and the high level of sensitivity for CIN3 despite the misclassification of HPV, we believe, further show the robustness of p16^{INK4a}. Finally, in our analysis, women with inadequate or insufficient tissues were excluded. Although this is likely due to the small nature of these tissues, as with most immunohistochemistry procedures, the results are dependent on the nature of the histologic section.

In conclusion, the results of the present study indicate that p16^{INK4a} expression in tissues can be used to identify progressive cervical neoplasia and hypothesized to be HPV-transformed cells (20). As others have found, our cross-sectional data similarly indicate that p16^{INK4a} possesses potential as a marker for triage and potentially for screening. However, its potential as a prognostic marker will require detailed follow-up data for a larger group of women; studies focusing on follow-up of the heterogeneous group of women diagnosed with CIN1 will be of particular value. Although our results indicate variability in p16^{INK4a} immunostaining in LEEP specimens in which multiple blocks were able to be evaluated, there does seem to be a field effect. Therefore, future studies might compare p16^{INK4a} immunostaining on cytologic slides to immunostaining on histologic slides from the matching tissue blocks of the biopsy and correlate staining with cross-sectional and cumulative prospective outcomes. Given the aforementioned limitation of histologic sections, development of an ELISA-type assay format would improve utility of p16^{INK4a} further. Widespread use of p16^{INK4a} as a marker would benefit from improved and uniform sampling. These further studies are needed to ultimately determine whether clinical management should be modified based on a women's p16^{INK4a} staining result.

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